Human Lactoferrin Can Be Alternative to Antibiotics

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***Abstract*—**Human milk lactoferrin protects the newborn infant against infection until its own immunological protection mechanism is formed. As shown by the studies of its physiological functions, lactoferrin, in addition to its antimicrobial properties, has anti-inflammatory, detoxicant, antioxidant and anticancer activities. In adults, lactoferrin is produced by epithelial cells and neutrophil leukocytes. The use of lactoferrin isolated from donor milk has shown its therapeutic activity. The lactoferrin behavior in pathologic states is now investigated in order to define indications for its medical use, primarily in infection therapy. Biotechnology is facing the task of producing recombinant human lactoferrin. It is expected that physicians will get novel highly effective and biologically safe human lactoferrin-containing drugs as early as in the next decade.

***Keywords*—**Lactoferrin, recombinant protein, therapy, transgenesis

I. Introduction

When penicillin was discovered 80 years ago, many believed that it would put an end to human infectious diseases. The joy, however, soon gave place to deep concerns, when the majority of widespread microorganisms proved to be capable of genetic self-transformation, which resulted in the spread of penicillin-resistant bacteria. Medical practitioners also contributed to the disaster by excessive and indiscriminate use of penicillin. Penicillin—on sale in every pharmacy—became available for self-treatment.

The further history of antibiotics is a procession of unsuccessful attempts of their creators to cope with the decreasing antibiotic sensitivity of microorganisms. There is nothing unusual about microorganisms developing antibiotic resistance. It is a natural mechanism that allows them to struggle for existence. Penicillin, for example, is nothing else but a product of microorganisms. Such "hazardous wastes" are their weapons in interspecies competition. From this it is obvious that bacteria producing antibiotics against other microorganisms have their own genetic mechanisms of resistance to these antibiotics. Bacteria are short-living. They leave genetic material behind them and any other bacterium, even of another species, may take it up. The ability of rather distantly related microbial species to make use of advantageous genetic information ensures them invulnerability in their habitat. As was discovered later, not only can bacteria develop mechanisms of genetic resistance to antibiotics, but they can also exchange genetic material responsible for such resistance.

Manuscript received August 13, 2010. This work was supported by Russian-Belorussia Government Program and "Transgenebank".
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Researchers from the Paris Descartes University have recently found a new mechanism by which microorganisms can acquire resistance to various antibiotics. It is mediated by acetyltransferase, which has a readily modifiable active site capable of blocking antibiotic activity (1).

Antibiotic-resistant bacteria are extremely dangerous to man. The literature describes cases of "export" of such microorganisms to other countries. For example, antibiotic-resistant salmonellae first appeared in Europe and then were detected in the U.S.A. In Russia, vancomycin had long been successfully used against enterococci until a patient from the U.S.A. brought in a vancomycin-resistant enterococcus. A further uncontrolled selection of antibiotic-resistant microorganisms may be disastrous for humanity.

Antibiotics are not only used in medicine. Animal and poultry breeders routinely add antibiotics in feed, using them as growth stimulators. Nobody knows how much antibiotics we consume with our meals and what the consequences will be.

Extensive scientific research resulted in the development of new antibiotics with different indications. While having high therapeutic efficacy, antibiotics may also cause a number of more or less severe side effects in humans. The most common complications of antibiotic therapy are allergic reactions. The fact that limited or generalized skin lesions, vasomotor rhinitis and arthralgias occur not only in antibiotic-treated patients but also in people working at antibiotic producing plants makes the antibiotic production environmentally unfriendly.

The use of antibiotics often results in the elimination of sensitive saprophytic microorganisms in the human intestine, with their place being overtaken by antibiotic-resistant opportunistic bacteria and fungi: coli forms, Proteus, staphylococci, yeast-like fungi, etc. This may cause vitamin deficiencies because intestinal bacteria produce certain vitamins.

Despite the whole array of outstanding problems associated with their use, antibiotic treatment has become a routine clinical practice, which will hardly be abandoned in the foreseeable future.

Meanwhile, doctors are deeply concerned about how to treat infection in patients who do not tolerate antibiotics, which antibacterial agents to administer to the large high-risk group including intensive care patients, pregnant women, and children.

The world scientific community is unanimous in the opinion that lactoferrin, a bactericidal protein from human breast milk, can break the vicious circle of the antibiotic-related problems (2-5).

The primary physiological function of lactoferrin is to ensure antibacterial protection of newborns and adults

(6).

The first component of this effect is the bacteriostatic activity of lactoferrin, which is associated with its ability to deprive bacteria of iron they need and thereby inhibit their growth. The mechanism is as follows: lactoferrin is secreted as an iron-free form (apolactoferrin) (7) but can readily bind with iron, when necessary. In the intercellular space or mucosa, apolactoferrin ties up the iron required for the growth of pathogenic microflora (8). Most microorganisms lack effective genetic mechanisms to oppose lactoferrin. Only few pathogens can partially overcome this protective barrier by synthesizing biomolecules (siderophores) competing with lactoferrin for iron. Yet another way of getting iron can be used by microorganisms if they manage to bind the molecules of lactoferrin and transferrin and thus reduce their iron sequestration capacity. The lactoferrin receptors of microorganisms are called lactoferrin-binding proteins A and B (LbpA и LbpB) (9-11). The probability of these events increases as the level of free lactoferrin declines but can be cut short by adding its exogenous analog.

The second component of the antimicrobial action of lactoferrin is its bactericidal activity. And here things look absolutely black for microorganisms as the bactericidal activity of the protein is independent of its iron-binding capacity (12-14).

By binding to the pathogen membrane, lactoferrin fixes itself firmly to the surface of the bacterium and thereby reduces its resistance to lysozyme and other antibacterial factors (15). Eventually, the molecular mechanism of the lactoferrin bactericidal activity results in the membrane destruction of both Gram-positive and Gram-negative bacteria (16). Because antimicrobial activity is so easy to test, the list of lactoferrin-sensitive bacteria species is ever increasing.

Lactoferrin undergoes partial proteolysis. The resulting peptides (lactoferricins) have increased selective antibacterial activity against certain microorganisms, which is even higher than that of the native lactoferrin molecule. Not only do lactoferricins damage bacteria, but they also prevent bacteria from penetrating into human cells (17-20).

Discovered as early as in 1939, lactoferrin was called “red protein” for its color due to the iron oxides. It has been a subject of a thorough research ever since, revealing more and more new properties and mechanisms of action. LF is one of the few human proteins being the focus of special regular international conferences. Hundreds of scientific articles and monographs on LF are published every year (21-26). In particular, it has been established that lactoferrin has antiviral activity as well (27-28). As shown by extensive experimental studies, lactoferrin can bind to viral particles and thus prevent them from penetrating into cells; it can also affect the virus itself (29-32).

Lactoferrin and its derivatives were shown to have antifungal activity, which is manifested in inactivation of sporozoites, so that they become unable to infect cells, or in killing the phytopathogen by destroying its cell wall (33-36).

LF can suppress systemic inflammation by binding bacterial lipopolysaccharides that cause septic states, as well as by activating the synthesis of anti-inflammatory cytokines (interleukin-18, γ-interferon), and by activating cell protection systems (37).

Lactoferrin can inactivate various types of toxins, including chemical radicals, and is therefore a promising agent for the treatment of secondary intoxication associated with chemo- and radiotherapy in cancer patients. This would allow effective therapy without reducing drug and radiation doses (38).

As shown by a recent series of thorough studies, LF can directly affect cancer cells and inhibit their spread (39-40, 66).

Unlike antibiotics, LF does not damage the normal intestinal microflora; moreover, it directly activates the growth of Bifidum and Lactobacillus (42).

Of note, there has been a successful experience of using LF in combination with antibiotics to enhance their therapeutic effects (43-44).

Human LF has one more attractive feature: it is absolutely safe and has no contraindications either in pediatric, or in adult patients.

Animal studies with radioactive LF showed that injected into the blood stream of an animal the protein is accumulated in the liver and undergoes a complete hydrolysis to amino acids within 2 hours (45-47). The iron ions released in the process take part in erythropoiesis.

Thus, LF is a multifunctional bactericidal protein with marked antimicrobial, antiviral and antifungal activities and not only capable of directly acting on the cause of septic states, but also of activating the body defense mechanisms for elimination of the accompanying inflammatory processes.

The torrent of LF research is continuously joined by streamlets of clinical studies demonstrating its high therapeutic efficacy. A summary of these studies performed in Russia and other countries may help in defining the strategy of future LF uses. Besides, it would be good for medical specialists to get an insight into the current situation with the development of methods for the protein commercial production, which is the prerequisite of introducing human LF therapy into routine clinical practice.

II. LF Expression and Levels in Humans: Norm and Pathology

The first appearance of LF can be detected in the 2-4 cell fertilized embryo, before the blastocyst formation. Later, it appears in neutrophil leukocytes at the late stage of fetal formation and in epithelial cells of the digestive and respiratory systems (48).

In the adult, very high concentrations of LF are found in colostrums and milk. LF is also present in endocrine secretions, including tears, saliva and semen, i.e. it can

be considered as a product of the glandular cells of the respective epithelial tissues (see Table 1).

TABLE 1
LACTOFERRIN LEVELS IN HUMAN BREAST MILK, BLOOD, SECRETIONS AND CELLS

Biological fluid, cells	LF levels, [g/L]
Colostral breast milk	7
Mature breast milk	1-2
Tears	1.5-2.2
Seminal fluid	0.5-1
Cervical mucus	0.5-1
Nasal secretion	0.1
Saliva	0.005-0.01
Bile	0.01-0.04
Urine	0.00001-0.00003
Blood (normal)	0.00002- 0.001
Blood (inflammation)	0.001-0.2
Synovial fluid	0.1-0.8

(Adapted from 1-4)

The main source of serum (plasma) LF is neutrophil leucocytes (49-52).

According to most researchers (53), serum LF levels in healthy adult humans, as determined by radio- and enzyme immunoassays, vary from 0.13 to 1.62 μg/mL. Such variation is not only due to errors and different assay conditions of LF determination, but also due to sex-, age-related, ethnic and geographical specifics of the studied populations (54-55). This was confirmed by special studies performed by Russian researchers. For instance, the mean serum LF concentration was 1.05+0.21 μg/mL in healthy residents of the city of Astrakhan and only 0.26+0.02 in Muscovites (55).

The fact that LF is present in various bodily fluids and cells throughout the entire human life is indicative of the physiological significance of this bactericidal protein not only for the newborns but for adults as well. LF appears in highest concentrations wherever there is a need to defend the potential portals of entry: in the barrier epithelial cells of the lachrymal gland, gastrointestinal system and in the uterine cervix epithelium. The human reproductive system seems to be under special LF control since high concentrations of the protein are also present in semen (see Table 1).

Some authors suggest that the observed elevations of serum LF levels in patients with infections are also the result of the protein increased expression.

Since hyperthermia is one of the physiological signals activating the cascade of adjustment reactions resulting in the production of various inflammatory mediators and their interaction, studies are carried out to establish relationships between LF and elevation of human body temperature, interleukin production and inflammatory reactions (56-58). Therefore, LF may be thought of as an acute phase protein.

Clinicians are well aware that patients with antecedent viral infections are prone to bacterial complications. This phenomenon is consistent with the fact that patients with a congenital or acquired LF deficiency are more susceptible to secondary infections (59).

Serum LF assays in various diseases of humans are still performed for research purposes alone, yet our current level of knowledge convincingly shows that accurate LF measurements can be of certain diagnostic and prognostic value.

Increased LF concentrations are routinely found in the pancreatic secretion in patients with chronic calculous pancreatitis at the stage of protein plugging and the following formation of ductal stones. Elevated serum LF levels were observed in rheumatoid arthritis (60-61), cystic disease (62), as well as in lung cancer, gastrointestinal and mammary gland neoplasms (63-76).

The increase is probably due to excessive LF production either by the tumor cells themselves, or by neutrophils.

Physicians from the Omsk State Medical Academy and the Omsk Municipal Acute Care Hospital No. 1 conducted a comparative study of increased LF levels in sera and cerebrospinal fluid (CSF) from 30 patients with secondary meningitis or meningoencephalitis (seven of the patients died). The LF levels in the tested biological fluids correlated with the disease severity. In CFS, LF elevations over the normal range were observed more often and were more notable than in serum (in 23 (77%) vs. 16 (53%) patients, respectively)

Measurements of serum LF levels in patients with gastric and duodenal ulcers were performed at the State Medical Academy of Astrakhan. 125 male patients aged 18 to 60 years were included in the study. LF concentrations were measured in enzyme immunoassay. The control group included 20 healthy men. According to the findings, serum LF levels in male patients with peptic ulcer were 1.5 to 2 times as high as in controls and did not depend on the ulcer localization and severity.

Evidently, hyperlactoferrinemia associated with the acute phase of peptic ulcer disease is clinically and pathogenetically appropriate because of the presence of inflammation, necrosis and proliferation in the ulcerative lesion of the mucosa. Hyperlactoferrinemia was reversible during the ulcer scarring (77-78).

A comparative study of serum LF levels in adolescent patients with bronchial asthma and healthy adolescent controls was carried out at the Veliky Novgorod University (79). An analysis of data collected over a two-year observation period showed that LF concentration in patients with bronchial asthma was nearly twice as high as in controls (1348.8 ± 462.0 ng/mL vs. 769.3 ±137.0 ng/mL).

As another example of a similar kind, we may speak of the interesting study conducted by a research team from the Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences, in cooperation with physicians from Novosibirsk Okrug Military Clinical Hospital. They measured serum LF

levels in a total of 95 patients with viral hepatitis A, B or C. The serum LF levels in acute and chronic phases were 850±420 ng/mL, 780±580 ng/mL and 680±500 ng/ml, respectively. The serum LF levels were significantly reduced by the treatment and in some cases did not differ from the normal 160±50 ng/mL 2-3 months after discharge from hospital. The authors believe that hepatitis virus infection causes reduction of the number of functional receptors in the liver and, as a result, an uncontrolled increase of mean serum LF levels. A decrease in serum LF levels can therefore be a diagnostic test for liver function evaluation and prognosis of possible complications.

On the other hand, serum LF levels were decreased in patients with pancreatic cancer (80-82).

III. Clinical Uses of Human and Bovine Milk Lactoferrin

In the light of today's knowledge of LF bactericidal properties, the folk method of treating rhinitis and cold by intranasal instillation of human milk appears to be not without reason. Up to now, human milk remains the only available source of human LF for the development and studies of pilot batches of LF-containing drugs with various indications.

In Russia, pioneer research in this field was conducted at the P.A.Hertsen Moscow Cancer Research Institute. Based on human milk LF, original formulations were developed for a modified conservative treatment of cancer patients. Among them, human milk LF, 6 mg per gelatin bolus, was effectively used to treat and prevent adverse reactions in the mouth and esophagus in cancer patients subjected to radiotherapy or radio-chemotherapy. The preparation should be taken 6 boluses daily, for 10 days. Another preparation, Laprot (protector lactoferrin), is a potent antioxidant and detoxicant, also having antibacterial, anti-inflammatory and immunomodulating properties. Laprot is intended for both intracavitory and intravenous administration. The preparation is effective in patients with septic processes, complications of chemo- and radiotherapy, bilirubinemias of different etiology, hematological disorders. Laprot has successfully passed the first phase of clinical studies in a total of over 1000 patients and is protected by RF patient (83-89). One liter of human colostrums is enough to produce 30 to 35 doses of the intravenous formulation. Treatment of one patient usually requires 5 doses per course and up to 10 doses in severe phylogenic diseases. These medicinal preparations were manufactured at the pilot plant of the said cancer institute. Although the production method is rather simple, there still remains a hard-to-solve problem of human milk deficit.

The preparation named Lactoferrin was produced at the Scientific Research Institute of Experimental Tumor Diagnostics and Therapy. The preparation was isolated from donor human milk by ion-exchange HPLC and was administered intravenously to 20 patients (280 transfusions administered) with various diseases (hepatitis C, bronchial asthma, neurodermitis, infected wound site, etc.). A marked therapeutic effect was observed in some cases (90).

Specialists from the Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences, and the Novosibirsk State University used a multistage purification of human milk and obtained an LF fraction capable of cleaving DNA. The fraction inhibited cell growth in a murine fibroblast culture and in human cancer cell cultures. Human breast milk LF can be used in the gene therapy of temporary and chronic protein deficiencies, in cancer therapy (specific effects on tumor cells), treatment of infections (effective immunization methods), etc. as a vehicle for delivery of genetic material into human cells. The advantages of LF over the traditionally used ligands include its ability of binding to plasmid DNA, its stability to proteolysis, low initial LF levels in human plasma, the high rate of the protein uptake from the blood stream, and the LF ability to penetrate from the cell cytoplasm into the nucleus.

Researchers from the Institute of Experimental Medicine, the Russian Academy of Medical Sciences (St. Petersburg), demonstrated that human LF, both in its original form and conjugated with DNA-binding compounds, could mediate gene transfer in animal cells so that the transduced genes were able to express model proteins. For example, LF was successfully used to correct the impaired synthesis of dystrophin in a murine model of a severe hereditary disease, Duchenne's muscular dystrophy, and to ensure expression of the apolipoprotein A1 gene in liver cells of the rat (91).

In connection with this avenue of research, intensive studies of cell surface receptors for LF are being carried out. It has been established that lactoferrin can bind to human cells of many different types. Surface receptors for LF were found on the epithelial cells of mucous membranes, lymphocytes, neutrophils and monocytes. LF binding can be iron-dependent or not (92-93). Along with "classical" protein receptors, some types of cells express LF receptors of nucleic nature (94).

Bovine milk LF is of interest because this natural bactericidal protein can be commercially produced even now, without waiting for the production of recombinant human LF. Preparations of this kind are offered by several companies. A typical product is a lyophilized LF isolated both from cow's milk and colostrums taken within 24-36 hours after calving. Nutritional supplements, also called functional foods, are made of food raw materials and, strictly speaking, are not medical products. Therefore, their certification procedure is rather simple. Studies are under way to find out to what extent bovine milk LF can substitute for human milk LF.

Not long ago, the Moscow medical clinic Chastnaya Praktika (Private Practice) launched a special bovine LF-based treatment program for viral hepatitises. The

addition of LF to combination therapy of viral hepatitises reduced the treatment duration dramatically: from minimum half a year to four weeks. The patients' liver function tests (ALT and AST) returned to normal ranges. The hepatitis virus titers decreased by 3-4 orders of magnitude. LF decreased the toxic side effects and enhanced the efficacy of interferon therapy. The use of LF was especially beneficial for patients with previous unsuccessful interferon therapy. The clinic charged about 2,000 U.S. dollars for a 2-week treatment course for viral hepatitis. The program has been suspended until the clinic gets all necessary approvals. At the same time, the interest in LF treatment of viral liver disease has increased after the discovery of the protein inhibitory effect on hepatitis viruses (95-97).

A lot of publications are devoted to bovine LF-based treatment of mouth and teeth bacterial and viral infections, including parodontosis (98-100).

The American company NEWAYS, for example, has developed and patented TransFactor, a product containing bovine colostrums and lactoferrin concentrates. It is expected that TransFactor may be used in the treatment of immunodeficiencies, weakness, fatigue, ageing, as well as for prophylaxis of infections, treatment of musculoskeletal disorders, injuries and vitamin deficiencies. The manufacturing company recommends that TransFactor should be contraindicated in pregnancy and lactation; besides, the product is not recommended for children because it contains growth factors.

The Finnish company Hankintatukku Oy has also developed its colostrums-based product named Ternimax (101-103). Colostrum for its production is obtained during the first 24 hours after calving (two milkings), from European cows kept in ecologically safe environment. The colostrum is purified of fat and casein using a patented technology and freeze-dried. Following oral administration of the capsuled product, a significant portion of the colostrum concentrate retains its activity all the way down to the lower intestine and is not changed by digestive enzymes.

Clinical studies showed that bovine colostrum can effectively restore the altered intestinal bacterial flora.

The Japanese firm Morinada Milk Industry chose the easiest way: it produced tableted LF (Lactoferrin Original) isolated from cow's milk. The recommended daily dose is six 100 mg tablets.

Two Russian firms, NARVAC and NOVENERGO, have jointly developed a new veterinary product, Polyferrin-A, possessing immunomodulating, antiviral, regenerating, anti-inflammatory and antioxidant activities.

Polyferrin-A is administered to cats and dogs, intravenously or subcutaneously, in the dose of 1 mL per animal weighing 1 to 50 kg. It is recommended that antihistamine agents should be given prior to intravenous administration.

Poliferrin-A is successfully used by Moscow veterinary clinics.

IV. Human Milk Lactoferrin

The amniotic fluid of parturient women contains 4250±500 ng/mL of the bactericidal protein LF. For comparison, the LF levels range 440±100 ng/mL in their blood, 60±20 ng/mL in urine and 5±2 ng/mL in CSF. The antibacterial activity of human LF (LF concentration is 5-7 g/L in human colostrum and 1-2 g/L in human milk) is sufficient to protect the newborn's digestive system against infection. The nature has seen to it that the newborn baby receives a loading prophylactic dose of colostral LF with its first breast-feeding. The bactericidal action of LF starts from the infant's mouth cavity. In the first months of life, the infant's oral cavity is predominantly inhabited by aerobes and facultative aerobes: streptococci, mainly S.salivarius, lactobacteria, neisseria, haemophils and Candida species, with their maximum populations falling onto the 4th month of life. Teething is associated with radical changes in the qualitative composition of the microflora. Simultaneously, bacterial distribution and colonization take place in the oral cavity, yielding numerous microsystems with relatively stable bacterial populations. Human milk LF and salivary lysozyme jointly protect the infant's oral cavity. Early loss of milk teeth might affect the denture development and cause malocclusion.

In the early 20th century, breast-feeding patterns in Russia followed the fashion. Young mothers of nobility preferred to delegate breast-feeding of their babies to wet-nurses. Later on, the attitudes in the Russian society changed and mothers stopped to refuse voluntarily to breast-feed. This change of patterns was largely contributed by the educational efforts of Russian pediatricians who had collected convincing statistical evidence of high mortality rates due to intestinal infection in bottle-fed babies.

Soviet Russia did not have today's abundance of milk substitutes. So when a mother could not breast-feed, wet nursing or donor milk had to be used. In large cities, donor milk collection centers were set up, where donor milk was screened for bacterial contamination and pasteurized. Unfortunately, the demand was high above the supply. This was largely due to the increasing number of mothers with lactation problems. With the improvement of living standards, donation of excess breast milk has become unlucrative. Many Russians refrain from looking for donor milk via the Internet. The reasons are obvious. If a woman has to sell her breast milk, she can hardly provide herself with adequate nutrition. There is no guarantee that with the donor milk the child will not receive antibiotics, narcotic or other drugs taken by the donor, or allergic agents. Who can guarantee that the donor is not a carrier of hepatitis virus or HIV? One cannot be sure that the wet nurse leads a healthy life, eats appropriate food and observes elementary sanitary norms when pumping the milk.

Providing of infants with adequate breast-feeding is a global problem. As proposed by the Russian Health

Ministry Scientific and Practical Center for Breast-Feeding Promotion, restrictions have been imposed on the use of infant milk formulas for children under 1 year and on the advertisement of such products in the Russian maternity hospitals and women's health clinics. These measures will undoubtedly be favorable for the health of those children whose mothers have enough milk. The Institute of Nutrition, RAMS, now recommends that children should be breast-fed to the age of 2 years, rather than 1 year, as was recommended before. However, the bottle-fed children having to feed on milk formulas will remain in the same unenviable situation as before. Natural human milk proteins should necessarily be added to the animal milk and nutrient mixtures for infants. This primarily concerns those proteins that protect the practically sterile newborn against bacteria. Experts believe that the rate of gastroenteritis in bottle-fed infants can be reduced ten times with the use of LF.

As demonstrated by animal studies, the gastrointestinal system of a newborn animal fed on its mother's milk grows and develops more intensively than that of an animal fed on milk formulas, and this development can be stimulated by adding human LF. This finding suggests that LF not only acts as a bactericidal factor in the infant's gastrointestinal system but also as a cell growth one (104). According to Taiwan researchers, genetically modified mice with increased LF levels in milk grew 10 to 15% faster than control mice (105).

It is not difficult to calculate the amount of lactoferrin that should be used in formula feeding. The calculation can be based on the LF amounts received by breast-fed infants. Taking into account the high LF levels in human colostrum and their gradual decrease during the period of lactation, one can easily estimate the amount of LF the infant consumes over a week, month, and a year of life.

Considering the number of infants annually left worldwide without their mothers' milk, it is obvious that satisfaction of this demand would require a large-scale production of human milk LF.

Therefore it becomes clear why the marketing evaluation of the annual world demand for human LF, for formula feeding alone, is estimated in billions of U.S. dollars.

V. Recombinant Human Lactoferrin: Achievements and Prospects

The development of commercial production of recombinant human LF for creation of highly effective and safe drugs of new generation, as well as for the use in infant formula feeding is of paramount social importance and high economic attraction.

The global biotechnology has now three major competing approaches to production of recombinant human LF: in plants, microscopic fungi, and in transgenic animal milk. Although we are dealing here with genetically modified organisms, the resulting medical products cannot be considered as transgenic foods, because the only difference from the products' normal consumer properties is that they contain an additive of breast milk LF, a natural protein for humans. What is more, the use of genetically modified organisms is the only way to obtain the necessary amounts of active human proteins of a proper quality. Recombinant human proteins (interferons, insulin, blood coagulation factors, certain hormones, etc.) synthesized in microbial expression systems have already found wide use in medicine. However, microbial production has a lot of important shortcomings: insolubility of the final product, the absence of protein glycosylation mechanisms, hypersensitivity reactions in the patients, environmentally harmful production which is being gradually abandoned in a number of countries.

The efficiency of LF commercial production is also an important factor. Bovine milk contains as little as 0.02 g/L LF (106). As estimated by the Russian company MILBI, 400 metric tons cow's milk would yield as little as 17 kg LF. Such production consumes too much power and raw material. Yet, the demand for the protein is very high and the first production facilities of this kind were launched as early as in 1986.

The Japanese firm Nikken, for example, applies a rather complicated method of protein purification to produce its Lactoferrin Gold 1.8 and uses about 3 L cow's milk per capsule of the final product (60 mg). Each pack contains 30 capsules and its production consumes 100 liters cow's milk. The product is recommended for adults and children above four years of age and therefore does not help solve the problem of artificial feeding of infants.

Ventria Bioscience, a Californian biotech company, plans to produce lysozyme, LF and human serum albumin from transgenic rice grain. Supposedly, a drink made from such transgenic rice can treat infant diarrhea, an enteric disease which is one of the leading causes of infant death worldwide: 3.1 million fatal cases every year, over 8400 cases per day, mostly young children in developing countries (107).

A go-ahead has been received for a large-scale open planting of transgenic rice. However, there are still some more barriers to overcome before Ventria rice products can come into the market. It is necessary to exclude the possibilities of uncontrolled escape of transgenic plants into the environment and transgenic contamination of food.

As a precaution measure, Ventria has been ordered to plant its rice at least 480 km away from ordinary rice fields. In the U.S.A, one should also consider the risk of dissemination of seeds of genetically modified plants by tornadoes and other elemental disasters. There must be emergency plans in place in order to prevent the seed dispersal beyond permissible limits.

The United Stated Department of Agriculture (USDA) opened a forum for all those willing to share their opinions on this innovation. More than 20,000 comments came in but only 29 of them were in favor of the new crop.

Production of recombinant human lactoferrin in the milk of transgenic animals is very attractive in many respects, despite all the difficulties with the creation of transgenic animals. This technical task can be solved in different ways. The most common methods include microinjection of genetic material into the pronuclei of zygotes, transfer of genetically transformed nuclei of a generative or somatic cell into the egg cell. Besides, gene transfer can be mediated by retroviruses, as well as by sperm cells or spermatogoniums. Cloning allows reproducing the most valuable transgenic genotypes. The challenge is to create heritable DNA constructs, which would ensure high and stable production of biologically active recombinant LF identical to the natural protein of feminine milk.

Scientists from the Institute of Gene Biology, Russian Academy of Sciences, developed gene constructs possessing the said properties and differing from each other in the use of either cDNA or genomic DNA of human LF and different regulatory sequences. The gene constructs were evaluated in primary transgenic female mice of different generations. The study resulted in the isolation of a number of gene constructs, which ensured average production of >10 g human LF per liter of murine milk. Maximum production of human lactoferrin in the milk of transgenic mice obtained using one of the best gene constructs was 33.0 g/L and 40.0 g/L. For this construct, two males were randomly selected from a group of primary transgenic males and mated with normal females. For each of the two male mice, several transgenic daughters of the first generation (F1) were evaluated. The average lactoferrin content was 23.4 g/L in the milk of the first male's daughters, and 16.2 g/L in the milk of the second male's daughters. The highest individual concentrations of recombinant human LF in the milk of the second and third generations of transgenic daughters were 24.2, 27.0 and 28.5 g/L. The lines of transgenic mice were maintained to the sixth—seventh generation. The overall average production of recombinant LF was 14 g/L.

Special comparative studies confirmed that recombinant LF obtained from the milk of transgenic mice was identical to human milk lactoferrin (108).

VI. Conclusion

The wide range of the human LF useful properties provides ample scope for its clinical uses, first of all as a bactericidal agent. Food industry is willing to use human LF as a nutrient supplement to powdered infant formulas or whole milk.

In the Russian Federation, the Chief Medical Officer has approved human LF for use (without limitation of age) as a biologically active dietary supplement, with the exception of LF isolated from human tissues and fluids. Similar approvals have been granted by USDA and the relevant supervisory bodies of other countries. Getting the marketing approval for a new drug is a long multi-step produce, therefore drugs containing human LF from milk of transgenic animals will become available later than LF-containing nutritional supplements.

However, the standard technical documentation for human LF-containing dietary supplements must include their quality and safety characteristics, sanitary standards, requirements for meeting the standards in the process of production, storage, transportation and sale of the products, as well as packing and labeling specifications, expiry date, quality and safety control methods. All the above said sets the task of developing an international standard for human LF.

The prospects of industrial production of human LF are not quite clear.

The Houston-based company Agennix, which claims to be the world's leader in the production of recombinant human lactoferrin, has already produced several hundred kilograms of the product by fermentation of genetically modified mold fungi Aspergillus oryzae in accordance with GMP standards. The company owns 76 patents and 50 more pending patent applications protecting methods for obtaining recombinant human LF, its commercial production and clinical uses. Phase II clinical studies of the new product Talactoferrin are under way. They are focused on two aspects: anti-cancer and wound-healing activities.

The commercial production of human LF will be based in Italy, at the production facilities of the Dutch company DSM, the key partner of Agenix.

The Sacramento-based biopharmaceutical company Ventra Bioscience intends to market transgenic rice. It is expected that the price of genetically modified rice (its human LF content is 25% of dry weight) will be 360 U.S. dollars per 1000 kg, three times as high as that of normal rice grown in the U.S.A. The costs of human LF isolation will largely depend on the desired degree of its purity and the field of use. For example, the approximate cost of the extract for the food industry is estimated at 0.50 to 1.0 U.S. dollars per kg flour, whereas the GMP-produced lactoferrin must cost 5 to 10 U.S. dollars per g, with an annual output of 600 kg. The company owns five US patents, four patents in other countries and is awaiting decisions on twenty more patents in the field of protein expression.

Human LF could be produced from the milk of transgenic animals by Pharming Group, the holder of 36 patents on various aspects of transgenic technology and products from milk of transgenic animals. Moreover, Pharming has recently bought about 60 patents from PPL Therapeutics. Besides, this company is a member of a broad network of partnership with a lot of other pharmaceutical companies and it practices selling or otherwise granting licenses.

Reportedly, the company has a herd of cows descending from the transgenic bull Herman born many years ago. The animal had already get old and was euthanized. The company is clearly in no hurry to implement the project and even might have lost

commercial interest in it. The reason is clear. The bull's descendants cannot boast of high and stable expression of human LF. The LF expression in the milk of transgenic cows of different generations varied in a wide range of 0.3 to 2.8 g/L (109). Creation of new transgenic animals carrying the human lactoferrin gene was abandoned as a long and costly process requiring up to 500,000 U.S. dollars per primary transgenic animal. To generate herds of animals for commercial production of the recombinant human protein, it would be good to have a certain selection of transgenic stud animals with a high and predictably inherited expression of the protein of interest. Launching a large-scale production of human LF would be economically viable with the herd-average LF expression of at least 5 g/L milk.

Pharming reported about the successful start of clinical trials of human LF from transgenic cows' milk. The trials took place in Europe and the U.S.A and were designed to evaluate the potential for the use of LF in the treatment of bacterial infection, cardiovascular diseases, hepatitis C, and coagulation disorders. Preclinical evaluation of potential use of LF for asthma treatment was conducted in Great Britain and the U.S.A. Yet, in 2002, the company announced that the further development of the human LF project will proceed within the framework of strategic alliances and partnerships, and this policy somewhat delays the completion of the studies in the said areas. Pharming has lately signed an agreement with DSM Biologies for a limited production of human LF for clinical studies.

The implementation of programs that use protein drugs from the milk of transgenic cows is annoyingly dependent on the epidemics of bovine spongiform encephalopathy, foot-and-mouth disease and other epizooties that periodically occur on one continent or another and make it necessary to destroy large animal populations and impose a moratorium on the export of animal products. Another factor causing long delays in obtaining final results is the long period of generation change in this animal species. Perhaps for these reasons many projects of transgenic human-protein production, both for medical and commercial uses, are now based on goats whose gestation term is half that of the cow's and who have a stronger natural immunity to infection.

The first drug from the milk of transgenic she-goats was developed by GTC Biotherapeutics (U.S.A). The drug, AtRyn, is approved for the treatment of patients with deficiencies of antithrombin, a protein with anticoagulant properties. Antithrombin deficiency is a hereditary disorder caused by a defect of the gene responsible for the protein structure. Patients with this hereditary abnormality should receive life-long anticoagulant therapy to prevent thrombosis. However, patients on anticoagulants are at increased risk during surgical interventions or labor. In such cases, clinicians administer human antithrombin obtained from donor blood.

A herd of genetically modified goats producing human antithrombin-containing milk is maintained in Charlton, Massachussets. According to the developers, the milk obtained from one she-goat can be equivalent up to 90 blood donations. While 100 kg of the medicinal substance produced by cultivating mammalian cells in fermenters cost hundreds of millions of U.S. dollars, the cost of same amount produced by 150 genetically modified she-goats does not exceed several millions dollars. Since hereditary antithrombin deficiency is a rare disorder (one case per 3-5 thousand people), large sales volumes can hardly be expected. In Europe and the U.S.A, the market is as small as 50 million U.S. dollars. However, with a broader spectrum of AtRyn indications (e.g. burns, coronary by-pass surgery, sepsis, and bone marrow transplantation) the annual sales of the product may amount to 700 million U.S. dollars worldwide.

As recently reported by the U.S. National Academy of Sciences, PharmAthene, a company specializing, among other things, in chemical and biological defense, has created genetically modified goats producing a nerve gas antidote with milk. The group of chemical warfare nerve gases includes Sarin, Soman, Tabun, VX, and other gases. Sarin, for instance, was used in the Iran-Iraq war in the 1980s, as well as in the Aum Shinrikyo terrorist attacks in 1994 and 1995. The main route of exposure to a nerve gas is by inhalation. The inhaled gas enters the blood stream and affects the nerve system. The company has developed an antidote, which decomposes the gas to inactive moieties. The antidote can be used for direct protection, as well as for poisoning prevention and management.

The antidote active component is butyryl cholinesterase, a difficult-to-synthesize enzyme present in minute concentrations in human blood. At different times attempts were made to obtain the enzyme from insects, yeast, bacteria, and other organisms, but always with a negligible yield. Researchers from PharmAthene have modified the goat genome by introducing the human gene responsible for the production of butyryl cholinesterase. This does not affect the animal's health and one liter of the goat milk is enough to produce two to three grams of butyryl cholinesterase.

The U.S. Department of Defense has allocated 213 million U.S. dollars for the project.

A lot of other transgenic goat milk-based drug development programs are currently at different stages of implementation. This is a vivid demonstration of the establishment of a new-type pharmaceutical industry based on the use of bioactive regulatory human proteins isolated from the milk of transgenic animals.

At the same time, there is an established market of LF produced from cow's milk. The world's annual output of the protein is now about 100 metric tons, with its market price reaching 300 U.S. dollars per kg. With a larger output volume, the price of bovine milk LF might decrease. According to some reports, Fontena, a

New Zealand-based multinational company specializing in diary products (its best known brand is the Anchor butter), has opened a facility for production of bovine LF. The construction cost 15 million U.S. dollars. The company states that the production will be targeted at satisfying the growing demand for LF in Japan, Korea, China and Taiwan. According to available information, over 75% of the world's produced bovine LF is now bought by Japan and South Korea, where the protein is added to infant food. The lopsidedness of the LF consumer market toward the Asian countries will hardly last for long, as there are already signs of activation on the European and U.S. markets. Naturally, this will result in a considerable expansion of the entire LF market.

It is so far too early to predict the future volumes of the world's LF production and pricing because they will largely depend on the outcome of the ongoing clinical studies of the protein.

Should lactoferrin be marketed as a bioactive additive for infant formula and as a drug substance with antimicrobial and immunomodulating properties, its future world's market may account for about 15 billion U.S. dollars per year. The developing sports nutrition industry is also interested in human LF.

It is quite reasonable to expect the development of new ophthalmologic products (eye drops for managing dry eyes), oral hygiene products (including those for parodontosis prevention and treatment), personal care products (shampoos, gels and soap for problem skin and hair). The world's LF market expansion due to these mass market products is estimated at 10 billion U.S. dollars per year.

If the LF efficacy in cancer management is confirmed and new LF-based anti-tumor agents appear, the expected volume of LF market may increase by another 19 billion U.S. dollars per year.

LF may be useful as a treatment and prophylactic agent in veterinary. Livestock farmers reckon that the creation of genetically modified farm animals (e.g., pigs with increased human LF levels in milk) will not only accelerate the growth of young animals, but will also prevent their mortality from diarrhea and anemia of infection. Today, in-feed antibiotics are used for this purpose. These antibiotics, however, not only have toxic effects on the animal health, but can also affect humans who eat such meat, making them allergic or unresponsive to antibiotic therapy. It is expected that transgenic cows with increased LF levels in milk will be less prone to mastitis.

The Russian program of human LF commercial production based on transgenic she-goats is aimed at producing the protein substance for the needs of the pharmaceutical industry and the use of human LF-containing whole goat milk in infant feeding. When assessing the prospective market for infant formulas and foods, including those containing human milk bactericidal proteins, we assume that the consumer will always prefer the traditional Russian product, goat milk, containing this dietary supplement. "Bovine lactoferrin is for calves, human milk lactoferrin is for infants." Drugs containing human LF must be biologically safe and non-allergenic. Their production must be environmentally friendly. We think that production of human LF in the milk of transgenic animals meets all these requirements.

The important fact is that we have made a breakthrough in gene construction and attained a high, economically significant LF expression in the milk of transgenic animals that persists across generations and is several times as high as in human breast milk. For the present time, this is quite a challenge in the case of human milk lactoferrin and lysozyme. Chinese scientists, for example, have recently obtained she-goats that can produce human lactoferrin of adequate quality but in concentrations as low as 0.765 g/L (110), and researchers from the University of California, Davis, have created transgenic animals producing lysozyme, but in concentrations 24% less than in human breast milk (111).

In October 2007, under the joint Russian-Byelorussian Program, we created first transgenic goats carrying the human LF gene.

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